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PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Group No.: 1645

Ulrich Certa, et al.

Serial No.: 10/735,973

Filed: December 15, 2003

For: **MODIFIED PHOSPHODIESTERASE POLYPEPTIDES WITH ALTERED PHYSIOCHEMICAL PROPERTIES**

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March 30, 2005

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Dear Sir:

Attached please find the certified copy of the foreign application from which priority is claimed for this case:

Country	Application No.	Filing Date
Europe	02028057.4	December 17, 2002

Respectfully submitted,

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The attached documents are exact copies of the European patent application described on the following page, as originally filed.

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr. | Patent application No. | Demande de brevet n°

02028057.4

Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
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R C van Dijk

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Bezeichnung der Erfindung/Title of the invention/Titre de l'invention:
(Falls die Bezeichnung der Erfindung nicht angegeben ist, siehe Beschreibung.
If no title is shown please refer to the description.
Si aucun titre n'est indiqué se referer à la description.)

Modified phosphodiesterase polypeptides with altered physicochemical properties

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Modified phosphodiesterase polypeptides with altered physicochemical properties

The invention relates to modified phosphodiesterase (PDE) polypeptides with altered physicochemical properties which are particularly useful in screening assays for the identification of agonists or antagonists of PDEs, the polynucleotides encoding such modified PDE polypeptides, antibodies against such polypeptides, methods of preparation and uses of such polypeptides.

The phosphodiesterases (PDEs) represent a family of enzymes that catalyze the hydrolysis of the various cyclic nucleoside monophosphates (including cAMP). These cyclic nucleotides have been found to act as second messengers within the cells by carrying impulses from cell surface receptors having bound various hormones and neurotransmitters to the inside of the cells. The task of phosphodiesterases is to degrade these cyclic mononucleotides once their messenger role is completed thereby regulating the level of cyclic nucleotides within the cells and maintaining cyclic nucleotide homeostasis.

Ten families of PDEs have been identified, designated PDE1 to PDE10. Within each family there are two or more related but distinct gene products (A, B, C, etc.) and for each of these alternative mRNA processing gives rise to multiple splice variants, identified by an additional arabic numeral in accordance with the most recent nomenclature recommendation (Molecular Pharmacology 46, 399-405, 1994).

HR/27.11.2002

All PDE gene products identified so far have one catalytic and a least one regulatory domain. The catalytic domain lies towards the carboxylic acid terminus of each PDE protein and has the greatest homology between the PDE families, being >75% homologous at the amino acid level (Perry and Higgs, Curr. Opinion Chem. Biol. 2, 472-481, 1998). Nevertheless, each of the more than 30 known PDEs have individually distinct substrate specificities, kinetic characteristics, regulatory properties and cellular and subcellular distributions (Houslay and Milligan, Trends in Biochem. Sci. 22, 217-224, 1997).

PDEs 4, 7 and 8 are highly specific for cAMP. PDEs 5, 6, 9 and 10 are selective for cGMP. PDE3s bind cAMP and cGMP with similar affinity, but hydrolyse cAMP most efficiently, cGMP rather poorly. PDE3s are, therefore, negatively regulated in their cAMP hydrolysing ability by cGMP. PDEs 1 and 2 hydrolyse both cAMP and cGMP, but with PDE1 the relative efficiencies vary with isoenzyme subtype (Perry and Higgs, Curr. Opinion Chem. Biol. 2, 472-481, 1998).

The amino terminal ends of PDEs consist of the regulatory domains, which are very different both between families and between variants within families. This region contains variously: a binding domain for Ca2+-calmodulin (CaM) in PDE1 ; non-catalytic cGMP-35 binding sites in PDEs 2, 5 and 6; a binding domain for the signalling G-protein transducin in PDE6. The amino terminal region also contains protein- and membrane-targeting sequences in several PDE3s and PDE4s, as well as protein kinase phosphorylation sites in PDEs 1, 3, 4 and 5. These phosphorylation sites are likely to be important in regulation of catalytic activity and/or subcellular location (Perry and Higgs, Curr. Opinion Chem. Biol. 2, 472-481, 1998).

Of particular interest to this invention are the PDE4 enzymes. PDE4 enzymes share a common structure, as deduced from their amino acid sequences (Beavo and Reifsnyder, Trends Pharmacol. Sci. 11, 150-155, 1990; Bolger at al., Mol. Cell Biol. 13, 6558-6571, 1993; Houslay, Sullivan and Bolger, Adv. Pharmacol. 44, 225-242, 1998). Members of each gene family (PDE4A, PDE4B, PDE4C, PDE4D) share common C-terminal regions, different for each family, and catalytic domains that for all PDE4 isoforms are very similar

(84% homology over about 360 amino acids across all PDE4s; Houslay, Sullivan and Bolger, Adv. Pharmacol. 44, 225-242, 1998).

From N-terminus to catalytic region, the sequence in "long form" PDE4s can be divided into 5 regions, three of which are isoform-specific (N-terminal region, linker regions 1 and 2, or LR1 and LR2) and two, more conserved regions, that are broadly similar between all isoforms, the upstream conserved regions 1 and 2 (UCR1 and UCR2). "Short form" PDE4s, e.g. PDE4A1, PDE4B2, PDE4D1, PDE4D2, lack UCR1 and LR1 plus differing amounts of the N-terminal region of UCR2. Throughout all regions are potential phosphorylation sites for a variety of kinases, including PKA (e.g. Ser 54 in human PDE4D3), mitogen activated protein kinases (e.g. Ser 487 of human PDE4B2), casein kinase II (e.g. Ser 489 of PDE4B2) and calcium-diacylglycerol dependent protein kinases (Houslay, Sullivan and Bolger, Adv. Pharmacol. 44, 225-242, 1998). Phosphorylations at some of these sites have been shown to activate the PDEs (e.g. Ser 54), others serve to inhibit. There is also evidence that some phosphorylations serve to prime the enzymes ready for subsequent activation by further phosphorylation at a different site or sites (Houslay, Sullivan and Bolger, Adv. Pharmacol. 44, 225-242, 1998). Other auto-regulatory sites may be found in the N-terminal sequence of certain PDE4s (Bolger at al., Mol. Cell Biol. 13, 6558-6571, 1993, Mc Phee et al., Biochem. J. 310, 965-974, 1995).

Specific PDE4 inhibition may be useful over a very wide range of disease areas. These include: asthma, atopic dermatitis, depression, reperfusion injury, septic shock, toxic shock, autoimmune diabetes, AIDS, Crohn's disease, multiple sclerosis, cerebral ischemia, psoriasis, allograft rejection, restenosis, stroke, ulcerative colitis, cachexia, cerebral malaria, allergic rhinoconjunctivitis, osteoarthritis, rheutmatoid arthritis, autoimmune encephalomyelitis (Houslay, Sullivan and Bolger, Adv. Pharmacol. 44, 225-242, 1998, WO02/074992). In the area of asthma, PDE4 inhibition helps to increase cAMP in bronchial smooth muscle, thereby producing a modest bronchodilatory effect, of use in the alleviation of asthmatic symptoms. But perhaps most importantly, inhibition of PDE4s is now a recognised method by which to suppress immune and inflammatory cell responses (Hughes et al. Drug Discov. Today 2, 89-101, 1997; Torphy, Am. J.Respir. Crit. Care Med. 157, 351-370, 1998; Teixeira et al., Trends Pharm. Sci. 18, 164-171, 1997).

PDE4s play major roles in modulating the activity of virtually every cell type involved in the inflammatory process. Immune and inflammatory conditions occur when recruitment of leukocytes from the blood compartment into tissues is either uncontrolled, inappropriate, prolonged or directed against self. In asthma, rheumatoid arthritis and multiple sclerosis, infiltration of tissues with inflammatory cells is prolonged and intense, leading ultimately to severe (and self-perpetuating) damage and loss of function. Acute disregulation of the immune system occurs in such conditions as acute respiratory distress syndrome (ARDS) where an overwhelming and generalised inflammatory response can frequently lead to death. There is also substantial evidence which suggests that inflammation may play a part in defining the extent of injury resulting from reperfusion following ischaemia, at least in brain and lung (Entman and Smith, Cardovasc. Res. 28, 1301-1311, 1994).

The PDE4D phosphodiesterases, which are the most preferred focus of the present invention, exist in mammals in the form of isoenzymes (which represent different molecular forms of the same enzyme polypeptide). These phosphodiesterases are localized in the cytosol of the cell and are unassociated with any known membraneous structures. The PDE4D isoenzymes specifically degrade cAMP and are a common target for such pharmacological agents as antidepressants (for example, rolipram). Several splice forms of PDE4D are known. the short forms, PDE4D1 and PDE4D2, lack UCR1 and LR1 sites. Rather than these, interest of the present invention focuses on the long isoforms, of which 6 are known, namely PDE4D3, PDE4D4, PDE4D5, PDE4D6, PDE4D7 and PDE4D8. All of these have in common the LR1 and UCR1 sites and the domains located at the C-terminus of these sites, but they have different N-terminal domains. Isoforms PDE4D6, D7 and D8 were recently disclosed in WO02/074992.

For identification of compounds that inhibit PDE4D isoenzymes, screening assays are used that are either whole cell assays in which the cells are treated with the inhibitor and cAMP hydrolysis is measured, or in vitro assays which use purified PDE4D isoenzymes either isolated from their natural sources or from cells overexpressing recombinant PDE4D isoenzymes. Such assays are described e.g. in WO00/09504, WO01/35979, WO01/68600 and WO98/45268. WO01/35979 describes the purification of recombinant PDE4D expressed in Saccharomyces Cerevisiae. The use of recombinant native forms of PDE4D for the identification of PDE agonists or antagonists has the

disadvantage that the PDE4D poypeptide does not have optimal physicochemical characteristics, especially with respect to aggregate formation. Aggregate formation can lead to protein precipitation and is known to potentially decrease the activity of enzymes, compared to the authentic oligomeric molecules. Thus, aggregation of the native forms of PDE4D polypeptides in screening assays aimed at identifying agonists or antagonists of PDE4D phosphodiesterase activity negatively affects the quality of these assays. In addition, the use of assays based on individual recombinant PDE4D isoforms has the disadvantage of potentially resulting in the identification of agonists or antagonists that are specific for a single isoform of a PDE4D only, instead of agonists or antagonists which act on all long or short form PDE4D isoforms together.

Figure 1: Specific phosphodiesterase activity for the different PDE4D isoforms D3 and D5 to D8, and the PDE4D core construct (DC).

Figure 2: Aggregate formation by different PDE4D isoforms and the core construct. In this figure, the gel filtration profiles of different isoforms of PDE4D (D3, D5, D6, D7, D8), and the core construct (DC) are shown. The first elution peak represents PDE4D aggregates. The pronounced shoulder in the elution profile of DC, where polypeptides of approx. 300,000 Dalton elute, indicates a decrease in aggregation of DC, as compared to the five isoforms of PDE4D.

Figure 3: Quantitation of aggregation of the core construct (DC) of PDE4D, and of the tubulin content of the core construct perparations. In A), the elution profile of DC is shown, with the fractions indicated on the bottom. B) shows an SDS-PAGE of the different fractions from the gelfiltration. C) shows the analysis of optical densities of individual fractions corresponding to the Coomassie stained SDS-PAGE in B).

Figure 4: Differences in aggregation and tubulin content of different isoforms and the core construct of PDE. A) SDS-PAGE gels are shown for DC and five isoforms of PDE4D which were analysed as described above. B) The percentage of non-aggregated PDE4D is shown for the core construct and the isoforms D3 and D5 to D8, based on total PDE4D protein. DC has the highest percentage of non-aggregated PDE4D, as compared to

the five isoforms. C) This figure shows the ratio between PDE4D and tubulin for DC and the isoforms D3 and D5 to D8, based on total PDE mass and total tubulin mass. As can be seen, the tubulin content of DC is significantly lower than that of the five PDE4D isoforms indicated.

Figure 5: Activity assay. Phosphodiesterase activity is shown both for the PDE4D core construct (DC) and PDE4D3 (D3).

Figure 6: Rolipram inhibition. Phosphodiesterase activity of the PDE4D core construct (DC) can be inhibited by Rolipram. The IC50 of DC phosphodiesterase activity by Rolipram inhibition was 0.34+/- 0.06 mM.

The term "polypeptide" as used herein, refers to a polymer of amino acids, and not to a specific length. Thus, peptides, oligopeptides and proteins are included within the definition of polypeptide.

The term "physicochemical properties" as used herein refers to the propensity of polypeptides to monomer formation, oligomer formation or aggregate formation.

The terms "aggregation" and "aggregate formation" as used herein are used interchangeably and refer to the type of association of polypeptide chains which, in contrast to oligomer formation, is an irreversible process and does not follow the law of mass interaction. Aggregation progresses in a time dependent manner and does not stop when a defined number of chains have associated. Aggregation of polypeptides can lead to protein precipitation because the molecular size of the aggregate is so dramatically increased that the solubility of the molecules is impaired. Aggregation is also known to potentially decrease the activity of the enzyme as compared to authentic oligomeric molecules.

The term "oligomer formation" as used herein refers to a reversible association of polypeptide chains whereby the ratio of oligomeric proteins and monomeric proteins at a certain protein concentration and at thermodynamic equilibrium is described by the association constant.

The term "non-aggregated" refers to polypeptides that are present either as oligomers or monomers or a mixture of oligomers and monomers at equilibrium according to the law of mass interaction.

The term "improved physicochemical properties" as used herein refers to a decrease in aggregate formation of polypeptides. For the purpose of definition, aggregate formation of the polypeptides of this invention is determined by quantitation of the fractions of a gelfiltration experiment in which aggregated PDE elutes, and of the fractions in which non-aggregated PDE elutes. The degree of aggregation is calculated in percent of non-aggregated PDE based on total (aggregated and non-aggregated) PDE. For the purpose of the definition of aggregation, aggregated PDE is considered to elute in fractions 2 to 4, in which aggreagated molecules of at least 2 Mio Daltons elute, and non-aggregated PDE is considered to elute in fractions 5 to 11, corresponding to the fractions in which molecules of about 0.3 Mio Daltons elute, when the following conditions are used for gel filtration: 50 µl of Ni-NTA agarose purified isoform preparation are injected into a Superose 12 size exclusion column (type PC3.2/30; Amersham Pharmacia Biotech), equilibrated with 50 mM TrisHCl pH 7.7, 100 mM NaCl, 0.5 mM MgCl2 at a flow rate of 0.1 ml/min at 4°C. Chromatograms are recorded at 278 nm. Starting from the elution volume onwards, the column eluate is collected as 50 µl fractions.

The term "long form PDE" as used herein refers to PDE isoforms which comprise an isoform specific N-terminal domain, linker regions LR1 and/or LR2, and upstream conserved regions UCR1 and UCR2.

The term "UCR1 start" as used herein refers to the first amino acid of the UCR1 domain of PDE4D, as annotated in SEQ ID No. 1 to 7.

The term "LF1 site" as used herein refers to the first amino acid of the splice site of PDE4D as indicated in SEQ ID No. 1-7.

The term "amino acids upstream of LF1" refers to the position of an amino acid counted from the LF-1 site towards the amino terminus of the native protein.

The term "D3" as used herein refers to the PDE4D3 isoform. The term "D4" as used herein refers to the PDE4D4 isoform. The term "D5" as used herein refers to the PDE4D5 isoform. The term "D6" as used herein refers to the PDE4D6 isoform as provided by WO02/074992. The term "D7" as used herein refers to the PDE4D7 isoform as provided by WO02/074992. The term "D8" as used herein refers to the PDE4D8 isoform as provided by WO02/074992. The polypeptide sequences of the human PDE4D isoforms D3, D4, D5, D6, D7, and D8 are shown in SEQ ID No.1 and 3-7.

The term "Ser54" as used herein refers to the Ser residue in position 54 of PDE4D3, and to the Ser residue in the respective position of any of the other PDE polypeptides. The position corresponding to Ser54 of PDE4D3 is annotated in SEQ ID No. 1 to 7 for the different isoforms and the core sequence which is common to all the PDE4D isoforms.

The term "Ser579" as used herein refers to the Ser residue in position 579 of PDE4D3, and to the Ser residue in the respective position of any of the other PDE polypeptides. The position corresponding to Ser579 of PDE4D3 is annotated in SEQ ID No. 1 to 7 for the different isoforms and the core sequence which is common to all the PDE4D isoforms.

The term "tubulin association" as used herein refers to the tubulin content of purified PDE preparations.

As used herein, the term "agonist" also refers to activators, and the term "antagonist" also refers to inhibitors of PDE phosphodiesterase activity.

The disadvantage of native recombinant PDE polypeptides resides in the formation of aggregates of the polypeptides, which gives rise to sub-optimal conditions for the identification of modulators of PDE phosphodiesterase activity in screening assays since aggreagate formation is known to lead to polypeptide precipitation and to potentially impair activity of a polypeptide. The present invention is directed to the generation of PDE polypeptides with improved physicochemical properties which are particularly useful for the purpose of identifying agonists or antagonists of these enzymes.

The problem of aggregate formation of recombinant PDE polypeptides was overcome in the present invention by generating a polypeptide comprising a PDE polypeptide sequence with an amino-terminal deletion, said polypeptide exhibiting decreased aggregate formation, as shown in Figures 2 and 3. These polypeptides have a specific activity that is comparable to the specific activity of the native recombinant proteins, as is shown in Figure 1. In a preferred embodiment, the invention provides a polypeptide comprising a PDE polypeptide sequence with an amino-terminal deletion wherein the proportion of non-aggregated PDE in the total protein preparation is from 55 %, preferably from 60%, more preferably from 65%, most perferably from 68% to 100%, preferably to 90 %, more preferably to 80 %, most preferably to 70% of total protein, as determined by the quantitation of eluted fractions from a gel filtration of the PDE fractions. Quantitation is performed as follows: Equal volume aliquots of fractions from Size Exclusion Chromatography, run as hereinbefore described, are analyzed by SDS-PAGE (one gel per run and isoform, Figure 3). Optical densities of Coomassie stained PDE are integrated as follows: After electrophoresis the Coomassie stained polyacrylamide gel was imaged by a video imaging system (Figure 1). Optical densities of PDE bands were integrated using a Macintosh computer and the public domain software "NIH Image",

version 1.61 (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). The integrated arbitrary units per PDE band as returned by the software reflect the relative PDE concentrations within the original pools. Integrated densities from PDE bands of fractions 2-4 are added up, giving a relative amount of aggregated PDE, PDE_{agg} (Figure 3). These fractions contain molecules of at least 2 Mio Daltons. Similarly, integrated densities from PDE bands of fractions 5-11 are added up, giving a relative amount of non-aggregated PDE, $PDE_{non\text{-}agg}$. In fractions 5-11, molecules in the range of 0.3 Mio Daltons elute. Relative amount of total PDE, PDE_{total}, is given by the sum of PDE_{agg} and $PDE_{non\text{-}agg}$. The percentage of non-aggregated PDE is given by the ratio of $PDE_{non\text{-}agg}$ and PDE_{total}, multiplied by 100.

In a preferred embodiment of the present invention the PDE polypeptide sequence with an amino-terminal deletion is a long form PDE polypeptide sequence. In a more preferred embodiment, the PDE polypeptide sequence is a PDE4 polypeptide sequence, preferably a long form PDE4 polypeptide sequence. In a most preferred embodiment, the polypeptide sequence is a PDE4D polypeptide sequence, preferably a long form PDE4D polypeptide sequence.

Methods of generating amino-terminal deletion mutants of polypeptides are well known. Such deletion mutants can be generated by using site directed mutagenesis, e.g. by using PCR (Sambrook et al., Molecular Cloning: A Laboratory Manual (1989), Cold Spring Harbor Laboratory Press, New York, USA).

In one embodiment of the present invention, the polypeptide comprises a PDE polypeptide sequence starting between 25 amino acids, preferably between 20 amino acids, more preferably between 15 amino acids, even more perferably between 10 amino acids, most preferably between 5 amino acids upstream of LF1 and the LF1 site.

In another embodiment the PDE polypeptide sequence starts at any amino acid located between the LF1 splice site and the first amino acid of the UCR1 start of the native PDE polypeptide. In the case of the most preferred embodiment of the PDE polypeptide, PDE4D, these sites are identical in all of the long form PDE4D isoforms.

In another more preferred embodiment of the present invention, the amino-terminal deletion is such that the PDE polypeptide sequence starts at the LF1 splice site of the native PDE polypeptide. This PDE polypeptide sequence is referred to as the "core construct". The core construct spans a sequence of PDE which is shared by all isoforms. The core construct sequence of PDE4D is shown in SEQ ID No. 2.

In another more preferred embodiment of the present invention, the PDE polypeptide sequence start is located 13 amino acids upstream of the UCR1 start of the native PDE polypeptide. The location of this position in the long form PDE4D isoforms and the core construct of PDE4D is shown in SEQ ID No. 1-7.

The basis for the generation of the above mentioned PDE polypeptide with an amino-terminal deletion and improved physicochemical characteristics can be any isoform of PDE. For the most perferred embodiment the PDE polypeptide sequence is an isoform of PDE4D selected from the group consisting of D3, D4, D5, D6, D7, and D8. However, the present invention also extends to further isoforms of PDE that have not yet been identified.

Also of use to the present invention are mutants of the PDE polypeptide herein before described. Of particular interest is a PDE polypeptide sequence comprising one or more mutations of Serine residues. Preferably, said Serines are mutated to either Alanine or Aspartic acid. In a most preferred embodiment, said Serine residues are selected from the group consisting of Ser54 and Ser579. Such Serines represent targets for phosphorylation of PDE polypeptides. Phosphorylation of some of these sites has ben shown to activate or ihibit the phosphodiesterase activity of PDEs.

Methods of generating amino acid substitutions in polypeptides are well known to those skilled in the art of Molecular Biology, e.g. methods such as site directed mutagenesis as described in Sambrook et al., Molecular Cloning: A Laboratory Manual (1989), Cold Spring Harbor Laboratory Press, New York, USA.

In another preferred embodiment of the present invention, the PDE polypeptide hereinbefore described exhibits decreased tubulin association, thus allowing to obtain purer PDE polypeptide preparations. To determine the decrease in tubulin association, the ratio of PDE molecules per tubulin molecules in the preparation of PDE polypeptide is calculated by quantitation of SDS-PAGE. Preferably, the ratio of PDE molecules per tubulin molecules is at least 5, preferably at least 10, more preferably at least 15, even more preferably at least 20, most preferably at least 25 molecules of PDE per molecules of tubulin. The tubulin content of PDE preparations is determined as follows: Equal volume aliquots of fractions from SEC are analyzed by SDS-PAGE (one gel per run and isoform, Figure 3). Optical densities of Coomassie stained PDE and tubulin bands are integrated as described hereinbefore for PDE aggreagation quantitation. Integrated densities from PDE bands of fractions 2-11 are added up, giving a relative amount of total PDE, PDE_{total}. Likewise, integrated densities from tubulin bands of fractions 2-11 are added up, giving $tubulin_{2-11}$, and thereby the relative amount of total tubulin, $tubulin_{total}$. The ratio of PDE and tubulin (Figure 4C) is identical with the ratio of the relative amount of total PDE4D, PDE_{tot}, and relative amount of total tubulin, $tubulin_{total}$, on a mass per mass basis.

The polypeptide of this invention includes polypeptides that are substantially homologous or identical to the polypeptides shown in SEQ ID No. 1-7 and have, in their native form, the phosphodiesterase activity and specificity of PDE. In particular, the polypeptide of this invention also includes the previously published polypeptide sequences of PDE4D3 to D8 as defined in U50159 (D3), L20969 (D4), S:1059276 (D5) and the sequences for D6, D7 and D8 as disclosed in WO02/074992. As used herein, two polypeptides (or a region of the polypeptides) are substantially homologous or identical when the amino acid sequences are at least 45-55%, typically at least 70-75%, more typically at least 80-85%, and most typically greater than 90% or more homologous or identical. To determine the percent homology or identity of two amino acid sequences, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in the sequence of one polypeptide for optimal alignment with the other polypeptide or nucleic acid molecule). The amino acid residues or nucleotides at corresponding amino acid positions are then compared. When a position in one sequence is occupied by the same amino acid residue as the corresponding position in the other sequence, then the molecules are homologous at that position. As used herein, amino acid "homology" is equivalent to amino acid "identity". The percent homology between the two sequences is a

function of the number of identical positions shared by the sequences *(i.e.,* percent homology equals the number of identical positions/total number of positions times 100). The PDE polypeptide sequences of the present invention are preferably mammalian, most preferably human. The present invention also includes variants of the polypeptides hereinbefore described with deletions, insertions, inversions, repeats, and substitutions selected according to general rules known in the art so as to have little effect on activity. For example, guidance concerning how to make phenotypically silent amino acid substitutions is provided in Bowie, Science 247 (1990), 1306-1310, wherein the authors indicate that there are two main strategies for studying the tolerance of an amino acid sequence to change.

The present invention provides polynucleotides that comprise polynucleotide sequences that encode the PDE polypeptide sequences hereinbefore described. As an example for a sequence comprised in such a polynucleotide, the polynucleotide sequence encoding the "core construct" of PDE4D is shown in SEQ ID No. 8. However, the present invention relates to all polynucleotides comprising polynucleotide sequences encoding any of the PDE polypeptide sequences hereinbefore described.

The present invention provides an expression vector or virus comprising a polynucleotide sequence that encodes the PDE polypeptide sequences hereinbefore described and which is capable of directing expression of the polynucleotide sequence in a compatible prokaryotic or eukaryotic host cell. The invention also provides a prokaryotic or eukaryotic host cell transformed or infected with such an expression vector or virus. The invention further provides a process for the production of such a polypeptide comprising culturing the host cell expressing the PDE polypeptide sequences hereinbefore described in a suitable medium so that said polypeptide is expressed, and purifying said polypeptide from the cells. In a preferred embodiment, the virus used for the expression of a modified PDE polypeptide is a recombinant baculovirus. Methods of generating recombinant baculoviruses for expression of recombinant proteins are well known to the man skilled in the art. Preferably, the recombinant baculovirus is based on pFASTBac as the transfer vector.The host cell of a preferred embodiment of the invention is an insect cell. Insect cells that can be infected with recombinant baculoviruses for the purpose of expressing recombinant proteins are well known in the art. The preferred process for the production of a modified PDE polypeptide is a process comprising culturing the insect cell

expressing the PDE polypeptide sequences hereinbefore described in a suitable medium so that said polypeptide is expressed, and purifying said polypeptide from the insect cell. To facilitate purification, the PDE polypeptide may also comprise a tag. Different types of such tags and the methods to join them with polynucleotide sequences encoding a polypeptide are well known to the person skilled in the art, e.g. myc-tag, FLAG-tag, six Histidine tags (hereinafter referred to as 6xHis tag). A review on tagging of proteins can be found e.g. in Fritze and Anderson, Methods in Enzymology (2000) 327, 3-16. It is well known that such tags may be joined to either side of the polypeptide. Some tags are known which may also be removed enzymatically from the purified polypeptide, e.g. FLAG-tag fused to the amino-terminus of a protein may be removed by digestion with bovine enterokinase (Hopp, T.P.(1988) Biotechnology, 6, 1204-1210). In a preferred embodiment the PDE polypeptides hereinbefore described are linked at their C-terminus to a 6xHis tag. The use of such 6xHis tags for ease of purification of recombinant polypeptides, and methods of purification of 6xHis tagged polypeptides are well known (e.g. in Sisk et al. (1994) J. Virol. 68, 766, and Petty, K.J. (1996) in: Ausubel, F.M. et al., eds. Current Protocols in Molecular Biology, Vol. 2, New York: John Wiley and Sons).

In a preferred embodiment, the polypeptide of the present invention is purified. As used herein, a polypeptide is said to be "purified" when it is substantially free of cellular material when it it isolated from recombinant and non-recombinant cells. A polypeptide, however, can be joined to another polypeptide with which it is not normally associated in a cell (e.g. in a "fusion protein") and still be "purified". Preferably, the polypeptides of the present invention are purified. The polypeptides of the invention can be purified to homogeneity. It is understood, however, that preparations in which the polypeptide is not purified to homogeneity are useful. The critical feature is that the preparation allows for the desired function of the polypeptide, even in the presence of considerable amounts of other components. Thus, the invention encompasses various degrees of purity. In one embodiment, the language "substantially free of cellular material" includes preparations of the polypeptide having less than about 30% (by dry weight) other proteins (i.e., contaminating protein), less than about 20% other proteins, less than about 10% other proteins, or less than about 5% other proteins. When a polypeptide is recombinantly produced, it can also be substantially free of culture medium, i.e., culture medium represents less than about 20%, less than about l0%, or less than about 5% of the volume of the polypeptide preparation.

The invention further provides an antibody against any polypeptide hereinbefore described. Preferably, the antibody binds to an epitope of the N-terminal domain of the PDE polypeptides. In another preferred embodiment the antibody binds to the N-terminus of a PDE4D polypeptide selected from the group consisting of D3, D4, D5, D6, D7, and D8. Such antibodies may be useful for different purposes. They may be used for detection of the polypeptides of this invention. They may also be used for affinity purification of the polypeptides of the present invention. Furthermore, they may be used in a screening assay to identify agonists or antagonists of PDE4D phosphodiesterase activity.

The polypeptides of the present invention can be used for a process of identifying and obtaining a drug candidate for therapy of a vascular disorder, said process comprising measuring the activation or inhibition of the phosphodiesterase activity of any of the above mentioned polypeptides. Different methods for measuring phosphodiesterase activity are known, eg. from Owens et al., Biochem. J. (1997) 326, 53-60. Such a drug candidate may be a compound that is either an agonist or an antagonist of the phosphodiesterase activity of PDE.

The present invention provides a pharmaceutical composition comprising an activator or inhibitor of the phosphodiesterase activity of the PDE polypeptide of the present invention, and a pharmaceutically acceptable carrier. Any conventional carrier material can be utilized. The carrier material can be an organic or inorganic one suitable for eteral, percutaneous or parenteral administration. Suitable carriers include water, gelatin, gum arabic, lactose, starch, magnesium stearate, talc, vegetable oils, polyalkylene-glycols, petroleum jelly and the like. Furthermore, the pharmaceutical preparations may contain other pharmaceutically active agents. Additional additives such as flavoring agents, stabilizers, emulsifying agents, buffers and the like may be added in accordance with accepted practices of pharmaceutical compounding.

An agonist or antagonist of the phosphodiesterase activity of the PDE polypeptide of the present invention or a pharmaceutically acceptable salt thereof may be used for the preparation of a medicament for the treatment of diseases caused by elevated or reduced phosphodiesterase activities. Such diseases include asthma, atopic dermatitis, depression,

septic shock, toxic shock, autoimmune diabetes, AIDS, Crohn's disease, multiple sclerosis, psoriasis, allograft rejection, ulcerative colitis, cachexia, cerebral malaria, allergic rhinoconjunctivitis, osteoarthritis, rheutmatoid arthritis, autoimmune encephalomyelitis, and vascular diseases such as reperfusion injury, cerebral ischemia, restenosis, stroke or peripheral arterial occlusive disease. The invention is, however, not limited to the diseases listed hereinbefore, but extends to all diseases that depend on elevated or reduced phosphodiesterase activity. Of particular interest to this invention is the use of such an agonist or antagonist of phosphodiesterase activity of PDE or a pharmaceutically acceptable salt thereof for the preparation of a medicament for the treatment of a vascular disease. The most preferred embodiment is the use of such agonist or antagonist of the phosphodiesterase activity of PDE or a pharmaceutically acceptable salt thereof for the preparation of a medicament for the treatment of stroke.

The present invention also provides a screening assay for the identification of agonists or antagonists of phosphodiesterase activity comprising at least one of the above described polypeptides. In addition, the polypeptides hereinbefore described can be used for crystallization. Crystals of the polypeptides are useful for assaying the interaction of compounds with phosphodiesterases. Thus, the present invention also provides a method of assaying a candidate agonist or antagonist for its ability to interact with a phosphodiesterase comprising crystallizing the polypeptide of claims 1 to 13 in a condition suitable for X-ray crystallography and conducting said X-ray crystallography on said polypeptide.

Furthermore, the present invention provides a kit for identifying agonists or antagonists of phosphodiesterase activity comprising at least one of the polypeptides hereinbefore described.

In the present invention, the products, methods and uses substantially as herein before described, especially with reference to the foregoing examples, are also claimed.

The invention will be further described by the following non-limiting examples.

Examples

In the following the term "isoform" relates to the natural PDE4D splice variants D3 and D5-D8 and the invented core variant, DC.

Cloning of the PDE4D isoforms 3-8 and the core construct

Core construct:

A cDNA encoding the core fragment that is common for all the PDE4D isoforms 3-8 starting with the amino acid sequence FDV carboxyterminal to the LF1 splice site was generated by PCR using a 5' oligonucleotide with a HindIII cloning site (gatgaattcaagctttttgatgtggacaatggcaca) introducing two additional amino acid (K and L) in front of the FDV sequence. At the 3' end a set of primers was used that either generated the native sequence (gtgatatctcattatcacgtgtcaggagaacgatcatctatgaca) or added a sequence encoding 6xHis residues (gtgatatctcattatcaa tgggatggtgatggtgcgtgtcaggagaacgatcatctatgac) to enable rapid purification of the recombinant proteins. The cDNA encoding the core construct was cloned as a EcoRI-EcoRV fragment into the expression vector pENTR^{TM}1a (GIBCO/BRL)

Isoforms (except core construct):

The DNA fragments encoding the isoform specific N-termini were generated by using synthetic oligonucleotides with terminal restriction enzyme sites for EcoRI and HindIII incorporated for directional cloning. These isoform specific DNA fragments were fused to the core construct sequence via the HindIII site introducing two additional amino acid residues (K and L). The integrity of the clones was confirmed by DNA sequencing prior to expression.

Expression of the PDE4D isoforms and the core construct in insect cells

The cDNAs were cloned into the pFASTBAC1 vector (Life Technologies. Inc) for expression in insect cells and the products were confirmed by sequencing. After recombination into the baculovirus genome the purified viral DNAs were transformed into the insect cells. Sf9 cells were cultured at 27^{0}C in TC100 medium (BioWhittaker) with

5% (v/v) fetal calf serum. Virus stocks were generated with a titer of 1.5x 10^9 pfu/ml. For large scale production of the isoforms 1-24 L fermentations of Sf9 cells were infected with a MOI of 1.

In one example, the 6xHis tagged PDE4D polypeptides DC, D3, D5, D6, D7 and D8 were produced in Sf9 cells in 1L spinner flasks using SF1 medium in the absence of serum. Infected cells were harvested 3 days after infection with the recombinant baculoviruses.

In another example, the PDE4D core construct DC was produced in a 24 L Airlifter Fermenter with 15 L medium (SF1 with 0% serum), 0.15 L lipids and 9 L Sf9 cells. During the entire fermentation procedure the cells were cultured at pH 6.2, 27.0+/- 0.2°C and a pO_2 of 30.0+/- 0.5 %. Cells were grown for 3 days. The cell number at infection was 2.3x10^6 cells/ml. Cells were infected with 450 ml recombinant baculovirus. Cells were harvested at 68h post infection and the cell pellet as well as the cconcentrated supernatant stored at -80°C until further processing.

Purification of 6xHis tagged PDE4D isoforms

Sf9 cells from 1 liter culture broth, overexpressing the respective isoform, were resuspended on ice in 50 ml 50 mM HEPES pH 7.8, 300 mM NaCl 10 mM imidazole, 1 mM DTT, supplemented with protease inhibitors (one protease inhibitor cocktail tablet "complete, EDTA-free"; Roche). Opening of cells was performed by use of a 50 ml Dounce homogenizator and the homogeneous mixture was centrifuged for 1 hour at 70'000 g and 4°C (Kontron TFT 45.94 rotor at 30'000 rpm). The supernate was filtrated through a filter with a pore size of 1.2 µM (Minisart; Sartorius, Germany) and then applied to a 6 ml Ni-NTA agarose column at 2 ml/min. After equilibration with 50 mM HEPES pH 7.8, 300 mM NaCl, 10 mM imidazole, protein was eluted with a linear 30 ml gradient from 10 to 230 mM imidazole in the same buffer. Fractions containing the PDE4D isoform as analyzed by Coomassie stained SDS-PAGE were pooled and stored frozen at –80°C. Fresh Ni-NTA agarose material was used for every different PDE4D isoform preparation in order to prevent cross contamination of isoforms.

Specific activities of 6xHis tagged PDE4D isoforms

Relative concentrations of Ni-NTA agarose purified isoform preparations were estimated by SDS-PAGE. Equal volume amounts of isoform preparations were applied to a gradient gel (4-12% NuPage; Invitrogen). After electrophoresis the Coomassie stained polyacrylamide gel was imaged by a video imaging system (Figure 1). Optical densities of PDE4D bands were integrated using a Macintosh computer and the public domain software "NIH Image", version 1.61 (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). The integrated arbitrary units per PDE4D band as returned by the software reflect the relative PDE4D concentrations within the original pools (Figure 1, red bars). Identities of PDE4D and tubulin bands had been verified by independent SDS-PAGE, excision of corresponding bands, trypsin cleavage and identification of tryptic peptides by MALDI-MS.

Activities of equal volume amounts of 10^6-fold diluted purified isoforms were determined by use of a commercial radioactive phosphodiesterase assay (cAMP-dependent phosphodiesterase [^{3}H] assay; Amersham Pharmacia Biotech), following the instructions of the manufacturer. The obtained arbitrary activity units reflect the relative PDE4D activities within the original pools (Figure 1, green bars).

Relative specific activities of PDE4D isoforms were calculated by dividing relative activity values by relative concentration values (Figure 1, blue bars).

Qualitative investigation of aggregation by size exclusion chromatography (SEC)

50 µl of Ni-NTA agarose purified isoform preparation was injected into a Superose 12 size exclusion column (type PC3.2/30; Amersham Pharmacia Biotech), equilibrated with 50 mM TrisHCl pH 7.7, 100 mM NaCl, 0.5 mM MgCl2 at a flow rate of 0.1 ml/min at 4°C. Chromatograms were recorded at 278 nm. Starting from the elution volume, the column eluate was collected as 50 µl fractions (Figure 2).

Quantification of aggregates and tubulin content

Equal volume aliquots of fractions from SEC were analyzed by SDS-PAGE (one gel per run and isoform, Figure 3). Optical densities of Coomassie stained PDE4D and tubulin bands were integrated as described above. Integrated densities from PDE4D bands of

fractions 2-4 were added up, giving a relative amount of aggregated PDE4D, PDE_{agg} (Figure 3). Similarly, integrated densities from PDE4D bands of fractions 5-11 were added up, giving a relative amount of non-aggregated PDE4D, $PDE_{non\text{-}agg}$. Relative amount of total PDE4D, PDE_{total}, is given by the sum of PDE_{agg} and $PDE_{non\text{-}agg}$. The percentage of non-aggregated PDE is given by the ratio of $PDE_{non\text{-}agg}$ and PDE_{total}, multiplied by 100 (Figure 4B).

Similarly, integrated densities from tubulin bands of fractions 2-11 were added up, giving $tubulin_{2\text{-}11}$, and thereby the relative amount of total tubulin, $tubulin_{total}$. The ratio of PDE and tubulin (Figure 4C) is identical with the ratio of relative amount of total PDE4D, PDE_{tot}, and relative amount of total tubulin, $tubulin_{total}$.

Activity assay and inhibition of phosphodiesterase activity

An IMAP FP-Assay was used for the determination of phosphodiesterase activity. The phosphodiesterase activity of the core contruct and PDE4D3 was measured using the HEFP Phosphodiesterase Assay Kit (Molecular Devices). 2 µl of PDE4D3 or PDE4D core construct, 2 µl of cAMP (to a final concentration of 40 nM) and 1 µl of test substance or carrier were incubated for 45 min on a shaker. 12 µl of Binding Solution provided by the kit (with beads diluted 1:320) were added, and the reaction mixture incubated on a shaker for 2 hours. Fluorescence polarisation of the samples was measured in a Packard BioScience Fusion a-FP HT using as an emission filter a Polarizer 535, and as an excitation filter, Fluorescein 485/20. Inhibition of the phosphodiesterase activity of the PDE4D core construct was determined using Rolipram as inhibitor, and using PDE4D core construct at 30 ng/ml.

Claims

1. A polypeptide comprising a PDE polypeptide sequence with an amino-terminal deletion, said polypeptide exhibiting decreased aggregate formation.

2. The polypeptide of claim 1, wherein said PDE polypeptide sequence is a long form PDE polypeptide sequence.

3. The polypeptide of claims 1 or 2, wherein said PDE polypeptide sequence is a PDE4 polypeptide sequence.

4. The polypeptide of any of claims 1 to 3 wherein said PDE polypeptide sequence is a PDE4D polypeptide sequence.

5. The polypeptide of any of claims 1 to 4 wherein the proportion of non-aggregated polypeptide in the total polypeptide preparation is from 55% to 100% of total polypeptide.

6. The polypeptide of any of claims 1 to 6, wherein the polypeptide sequence starts at any amino acid located between the LF1 splice site and the first amino acid of the UCR1 start of the native PDE polypeptide.

7. The polypeptide of any of claims 1 to 7, wherein the polypeptide sequence starts at the LF1 splice site of the native PDE polypeptide.

8. The polypeptide of any of claims 1 to 8, wherein the PDE polypeptide sequence start is located 13 amino acids upstream of the UCR1 start of the native PDE polypeptide.

9. The polypeptide of any of claims 4 to 7 wherein the PDE4D polypeptide sequence is a sequence of an isoform selected from the group consisting of D3, D4, D5, D6, D7, and D8.

10. The polypeptide of claims 1 to 9 wherein said polypeptide comprises one or more mutations of Serine residues.

11. The polypeptide of claim 10 wherein said Serine residues are mutated to either Alanine or Aspartic acid.

12. The polypeptide of claims 10 or 11 wherein said Serine residues are selected from the group consisting of Ser54 and Ser579.

13. The polypeptide of any one of claims 1 to 9 wherein said polypeptide exhibits decreased tubulin association.

14. A polynucleotide sequence encoding a polypeptide as claimed in claims 1 to 13.

15. An expression vector or virus comprising a polynucleotide sequence as claimed in claim 14 and being capable of directing expression of the polynucleotide sequence in a compatible prokaryotic or eukaryotic host cell.

16. A virus as claimed in claim 15 wherein the virus is a recombinant baculovirus.

17. A prokaryotic or eukaryotic host cell transformed or infected with the expression vector or virus of claims 15 or 16.

18. A host cell as claimed in claim 17, wherein the host cell is an insect cell.

19. A process for the production of a polypeptide as claimed in claims 1 to 13, comprising culturing the host cell of claims 15 or 16 in a suitable medium so that said polypeptide is expressed, and purifying said polypeptide from the cells.

20. A process for the production of a polypeptide as claimed in claim 19 wherein the host cell is an insect cell.

21. A polypeptide according to claims 1 to 13 produced by the process of claims 19 or 20.

22. An antibody against a polypeptide as claimed in claims 1 to 13.

23. An antibody as claimed in claim 22, wherein the antibody binds to an epitope of the N-terminal domain of the polypeptide of claims 1 to 13.

24. A process for identifying and obtaining a drug candidate for therapy of an inflammatory disease, said process comprising measuring the activation or inhibition of the phosphodiesterase activity of any of the polypeptides claimed in claims 1 to 13.

25. A compound identified according to the process of claim 24.

26. A pharmaceutical composition comprising an activator or inhibitor of the phosphodiesterase activity of the polypeptide claimed in any of the claims 1 to 13, and a pharmaceutically acceptable carrier.

27. Use of an activator or inhibitor of the phosphodiesterase activity of the polypeptide claimed in any of the claims 1 to 13 or a pharmaceutically acceptable salt thereof for the preparation of a medicament for the treatment of an inflammatory disease.

28. A screening assay for the identification of agonists or antagonists of phosphodiesterase activity comprising at least one of the polypeptides of claims 1 to 13.

29. Use of a polypeptide of claims 1 to 13 for crystallization.

30. A method of assaying a candidate agonist or antagonist for its ability to interact with a phosphodiesterase comprising crystallizing a polypeptide of claims 1 to 13 in a condition suitable for X-ray crystallography and conducting said X-ray crystallography on said polypeptide.

31. A kit for identifying agonists or antagonists of phosphodiesterase activity comprising at least one of the polypeptides of claims 1 to 13.

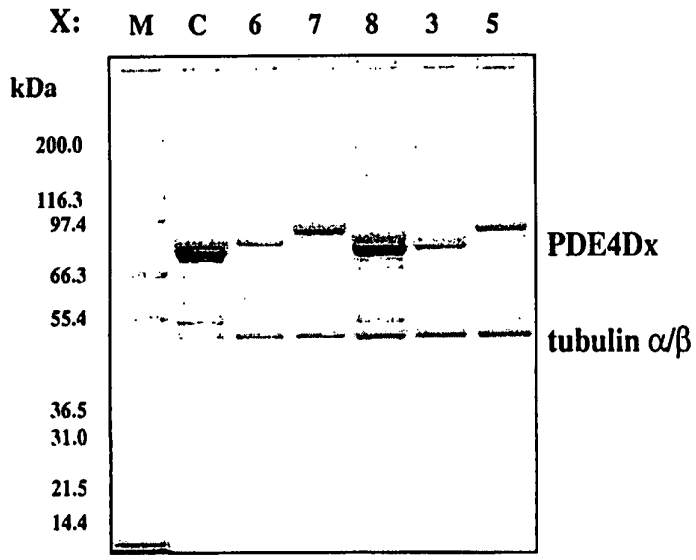
32. The products, methods and uses substantially as herein before described, especially with reference to the foregoing examples.

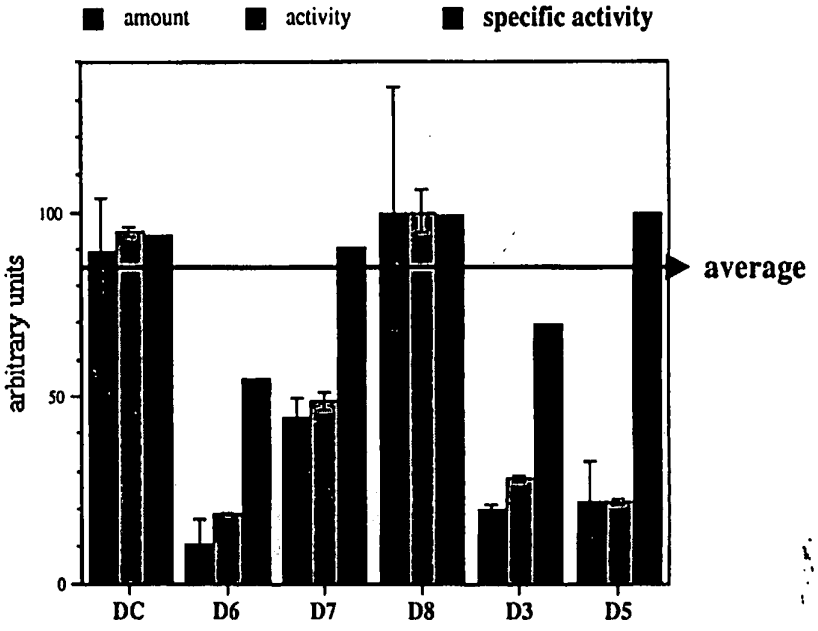
Abstract

Modified phosphodiesterase (PDE) polypeptides with altered physicochemical properties are disclosed, together with the polynucleotides encoding such modified PDE polypeptides, antibodies against such polypeptides and methods of preparation. Such polypeptides are useful for screening assays for identification of agonists or antagonists of phosphodiesterase activity.

***

Figure 1





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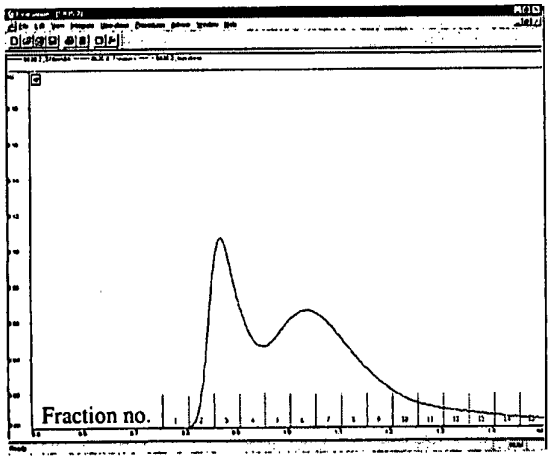

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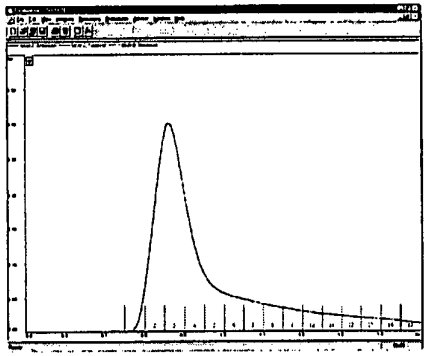

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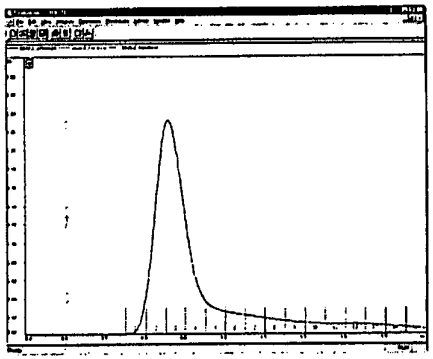

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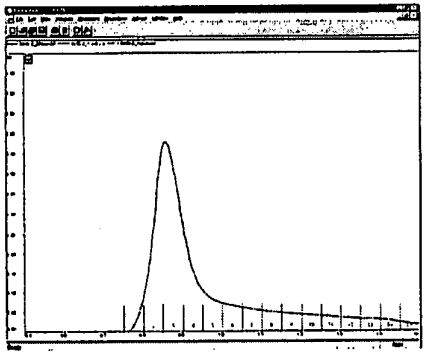

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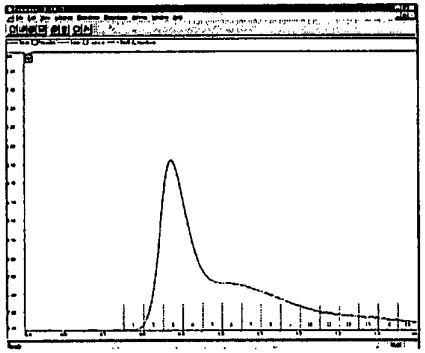

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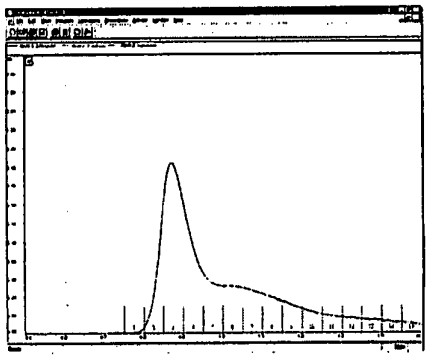

D8

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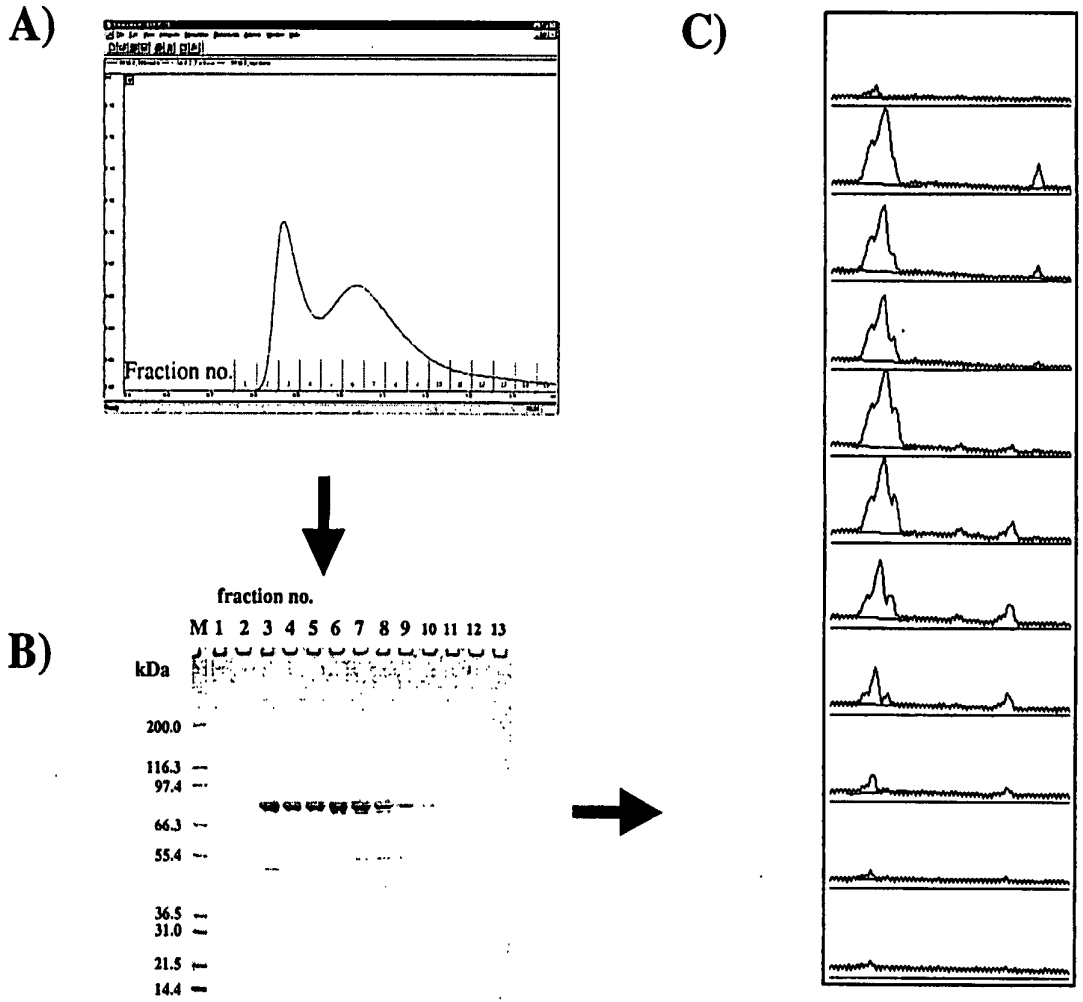
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fraction no.
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36.5
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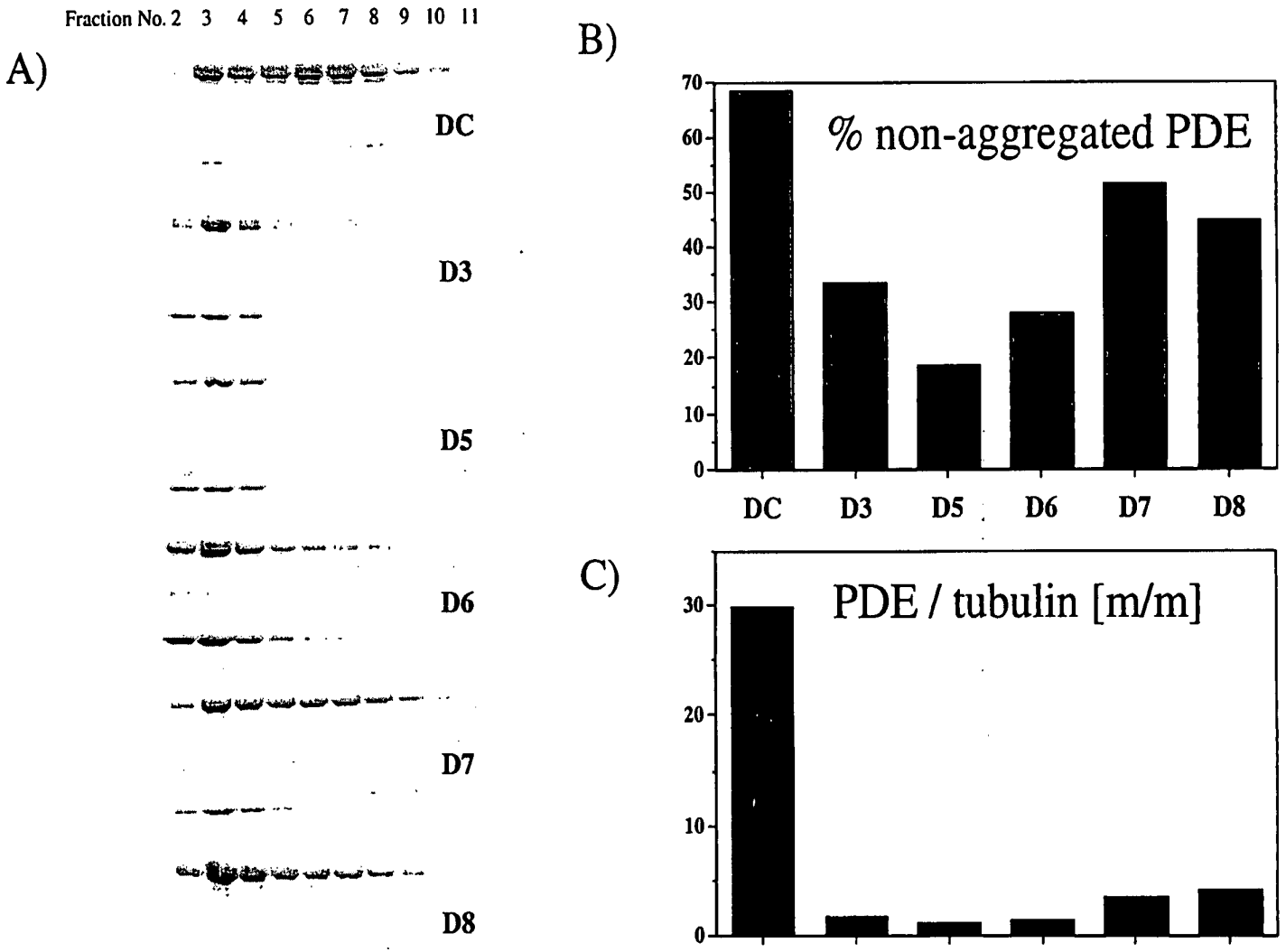
14.4
C)

Figure 4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D8
B)
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40
30
20
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D5
D6
D7
D8
C)
PDE / tubulin [m/m]
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20
10
0

Figure 5

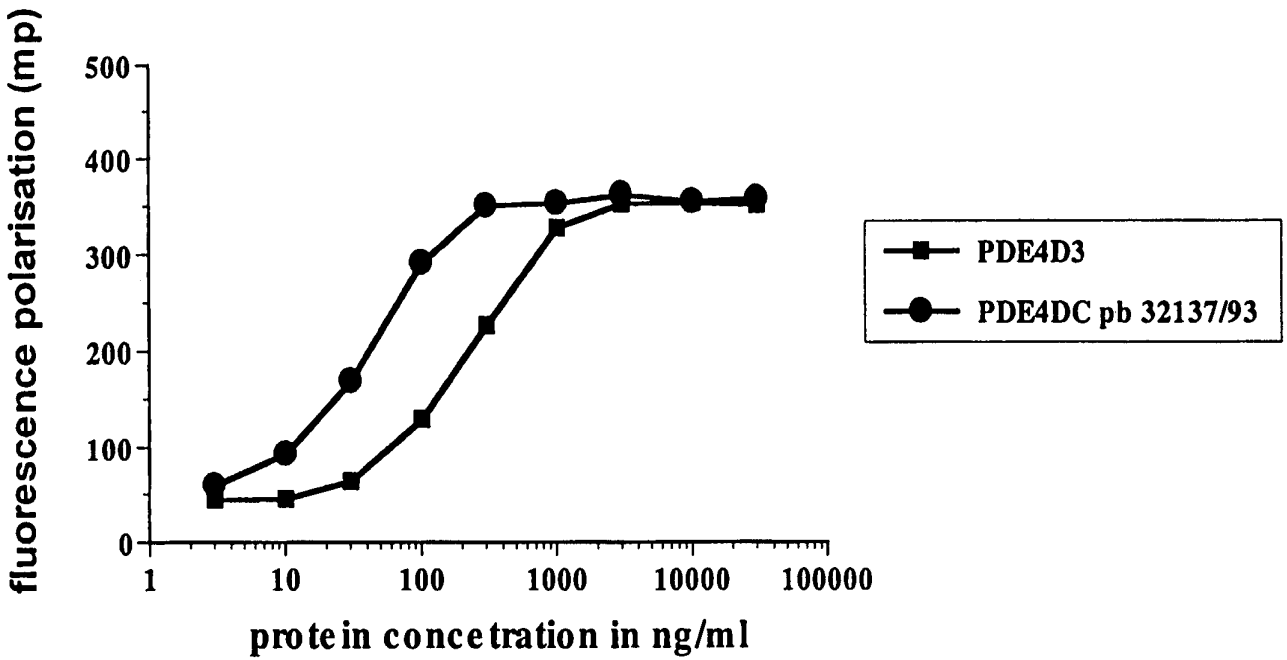
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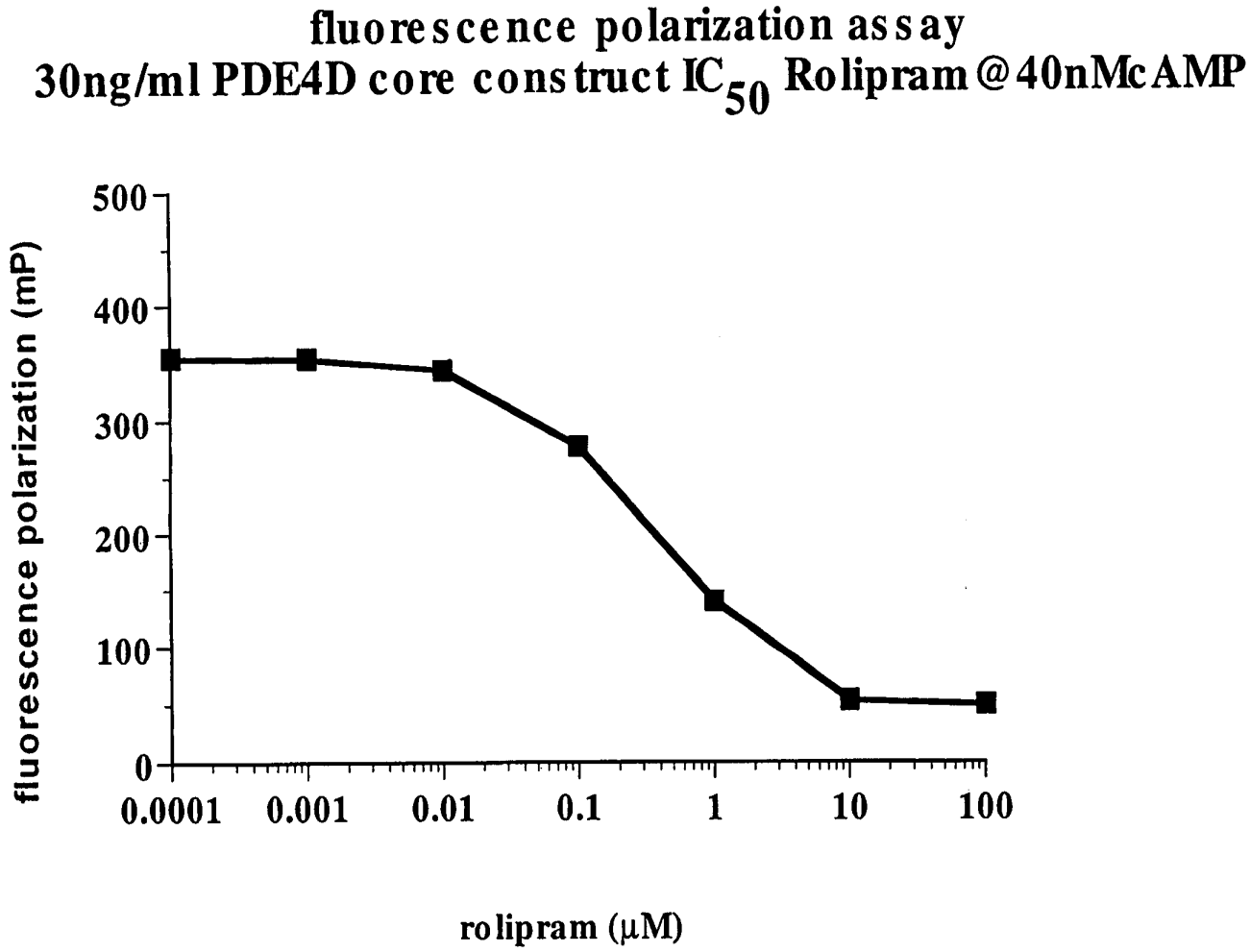
Figure 6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Asp His Pro Gly Val Ser Asn Gln Phe Leu Ile Asn Thr Asn Ser Glu

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385 390 395 400

Ala Val Gly Phe Lys Leu Leu Gln Glu Glu Asn Cys Asp Ile Phe Gln

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Asn Leu Thr Lys Lys Gln Arg Gln Ser Leu Arg Lys Met Val Ile Asp

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Asp Arg Ala Pro Ser Lys Arg Ser Pro Met Cys Asn Gln Pro Ser Ile
100 105 110

Asn Lys Ala Thr Ile Thr Glu Glu Ala Tyr Gln Lys Leu Ala Ser Glu
115 120 125

Thr Leu Glu Glu Leu Asp Trp Cys Leu Asp Gln Leu Glu Thr Leu Gln
130 135 140

Thr Arg His Ser Val Ser Glu Met Ala Ser Asn Lys Phe Lys Arg Met
145 150 155 160

Leu Asn Arg Glu Leu Thr His Leu Ser Glu Met Ser Arg Ser Gly Asn
165 170 175

Gln Val Ser Glu Phe Ile Ser Asn Thr Phe Leu Asp Lys Gln His Glu

180 185 190

Val Glu Ile Pro Ser Pro Thr Gln Lys Glu Lys Glu Lys Lys Lys Arg

195 200 205

Pro Met Ser Gln Ile Ser Gly Val Lys Lys Leu Met His Ser Ser Ser

210 215 220

Leu Thr Asn Ser Ser Ile Pro Arg Phe Gly Val Lys Thr Glu Gln Glu

225 230 235 240

Asp Val Leu Ala Lys Glu Leu Glu Asp Val Asn Lys Trp Gly Leu His

245 250 255

Val Phe Arg Ile Ala Glu Leu Ser Gly Asn Arg Pro Leu Thr Val Ile

260 265 270

Met His Thr Ile Phe Gln Glu Arg Asp Leu Leu Lys Thr Phe Lys Ile

275 280 285

Pro Val Asp Thr Leu Ile Thr Tyr Leu Met Thr Leu Glu Asp His Tyr

290 295 300

His Ala Asp Val Ala Tyr His Asn Asn Ile His Ala Ala Asp Val Val

305 310 315 320

Gln Ser Thr His Val Leu Leu Ser Thr Pro Ala Leu Glu Ala Val Phe

325 330 335

Thr Asp Leu Glu Ile Leu Ala Ala Ile Phe Ala Ser Ala Ile His Asp

340 345 350

Val Asp His Pro Gly Val Ser Asn Gln Phe Leu Ile Asn Thr Asn Ser

355 360 365

Glu Leu Ala Leu Met Tyr Asn Asp Ser Ser Val Leu Glu Asn His His

370 375 380

Leu Ala Val Gly Phe Lys Leu Leu Gln Glu Glu Asn Cys Asp Ile Phe
385 390 395 400
Gln Asn Leu Thr Lys Lys Gln Arg Gln Ser Leu Arg Lys Met Val Ile
405 410 415
Asp Ile Val Leu Ala Thr Asp Met Ser Lys His Met Asn Leu Leu Ala
420 425 430
Asp Leu Lys Thr Met Val Glu Thr Lys Lys Val Thr Ser Ser Gly Val
435 440 445
Leu Leu Leu Asp Asn Tyr Ser Asp Arg Ile Gln Val Leu Gln Asn Met
450 455 460
Val His Cys Ala Asp Leu Ser Asn Pro Thr Lys Pro Leu Gln Leu Tyr
465 470 475 480
Arg Gln Trp Thr Asp Arg Ile Met Glu Glu Phe Phe Arg Gln Gly Asp
485 490 495
Arg Glu Arg Glu Arg Gly Met Glu Ile Ser Pro Met Cys Asp Lys His
500 505 510
Asn Ala Ser Val Glu Lys Ser Gln Val Gly Phe Ile Asp Tyr Ile Val
515 520 525
His Pro Leu Trp Glu Thr Trp Ala Asp Leu Val His Pro Asp Ala Gln
530 535 540
Asp Ile Leu Asp Thr Leu Glu Asp Asn Arg Glu Trp Tyr Gln Ser Thr
545 550 555 560
Ile Pro Gln Ser Pro Ser Pro Ala Pro Asp Asp Pro Glu Glu Gly Arg
565 570 575
Gln Gly Gln Thr Glu Lys Phe Gln Phe Glu Leu Thr Leu Glu Glu Asp

580 585 590

Gly Glu Ser Asp Thr Glu Lys Asp Ser Gly Ser Gln Val Glu Glu Asp

595 600 605

Thr Ser Cys Ser Asp Ser Lys Thr Leu Cys Thr Gln Asp Ser Glu Ser

610 615 620

Thr Glu Ile Pro Leu Asp Glu Gln Val Glu Glu Glu Ala Val Gly Glu

625 630 635 640

Glu Glu Glu Ser Gln Pro Glu Ala Cys Val Ile Asp Asp Arg Ser Pro

645 650 655

Asp Thr His His His His His His

660

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Met Ala Gln Gln Thr Ser Pro Asp Thr Leu Thr Val Pro Glu Val Asp

1 5 10 15

Asn Pro His Cys Pro Asn Pro Trp Leu Asn Glu Asp Leu Val Lys Ser

20 25 30

Leu Arg Glu Asn Leu Leu Gln His Glu Lys Ser Lys Thr Ala Arg Lys

35 40 45

Ser Val Ser Pro Lys Leu Ser Pro Val Ile Ser Pro Arg Asn Ser Pro

50 55 60

Arg Leu Leu Arg Arg Met Leu Leu Ser Ser Asn Ile Pro Lys Gln Arg

65 70 75 80

Arg Phe Thr Val Ala His Thr Cys Lys Leu Phe Asp Val Asp Asn Gly

85 90 95

Thr Ser Ala Gly Arg Ser Pro Leu Asp Pro Met Thr Ser Pro Gly Ser

100 105 110

Gly Leu Ile Leu Gln Ala Asn Phe Val His Ser Gln Arg Arg Glu Ser

115 120 125

Phe Leu Tyr Arg Ser Asp Ser Asp Tyr Asp Leu Ser Pro Lys Ser Met

130 135 140

Ser Arg Asn Ser Ser Ile Ala Ser Asp Ile His Gly Asp Asp Leu Ile

145 150 155 160

Val Thr Pro Phe Ala Gln Val Leu Ala Ser Leu Arg Thr Val Arg Asn

165 170 175

Asn Phe Ala Ala Leu Thr Asn Leu Gln Asp Arg Ala Pro Ser Lys Arg

180 185 190

Ser Pro Met Cys Asn Gln Pro Ser Ile Asn Lys Ala Thr Ile Thr Glu

195 200 205

Glu Ala Tyr Gln Lys Leu Ala Ser Glu Thr Leu Glu Glu Leu Asp Trp

210 215 220

Cys Leu Asp Gln Leu Glu Thr Leu Gln Thr Arg His Ser Val Ser Glu

225 230 235 240

Met Ala Ser Asn Lys Phe Lys Arg Met Leu Asn Arg Glu Leu Thr His
245 250 255
Leu Ser Glu Met Ser Arg Ser Gly Asn Gln Val Ser Glu Phe Ile Ser
260 265 270
Asn Thr Phe Leu Asp Lys Gln His Glu Val Glu Ile Pro Ser Pro Thr
275 280 285
Gln Lys Glu Lys Glu Lys Lys Lys Arg Pro Met Ser Gln Ile Ser Gly
290 295 300
Val Lys Lys Leu Met His Ser Ser Ser Leu Thr Asn Ser Ser Ile Pro
305 310 315 320
Arg Phe Gly Val Lys Thr Glu Gln Glu Asp Val Leu Ala Lys Glu Leu
325 330 335
Glu Asp Val Asn Lys Trp Gly Leu His Val Phe Arg Ile Ala Glu Leu
340 345 350
Ser Gly Asn Arg Pro Leu Thr Val Ile Met His Thr Ile Phe Gln Glu
355 360 365
Arg Asp Leu Leu Lys Thr Phe Lys Ile Pro Val Asp Thr Leu Ile Thr
370 375 380
Tyr Leu Met Thr Leu Glu Asp His Tyr His Ala Asp Val Ala Tyr His
385 390 395 400
Asn Asn Ile His Ala Ala Asp Val Val Gln Ser Thr His Val Leu Leu
405 410 415
Ser Thr Pro Ala Leu Glu Ala Val Phe Thr Asp Leu Glu Ile Leu Ala
420 425 430
Ala Ile Phe Ala Ser Ala Ile His Asp Val Asp His Pro Gly Val Ser

435 440 445

Asn Gln Phe Leu Ile Asn Thr Asn Ser Glu Leu Ala Leu Met Tyr Asn

450 455 460

Asp Ser Ser Val Leu Glu Asn His His Leu Ala Val Gly Phe Lys Leu

465 470 475 480

Leu Gln Glu Glu Asn Cys Asp Ile Phe Gln Asn Leu Thr Lys Lys Gln

485 490 495

Arg Gln Ser Leu Arg Lys Met Val Ile Asp Ile Val Leu Ala Thr Asp

500 505 510

Met Ser Lys His Met Asn Leu Leu Ala Asp Leu Lys Thr Met Val Glu

515 520 525

Thr Lys Lys Val Thr Ser Ser Gly Val Leu Leu Leu Asp Asn Tyr Ser

530 535 540

Asp Arg Ile Gln Val Leu Gln Asn Met Val His Cys Ala Asp Leu Ser

545 550 555 560

Asn Pro Thr Lys Pro Leu Gln Leu Tyr Arg Gln Trp Thr Asp Arg Ile

565 570 575

Met Glu Glu Phe Phe Arg Gln Gly Asp Arg Glu Arg Glu Arg Gly Met

580 585 590

Glu Ile Ser Pro Met Cys Asp Lys His Asn Ala Ser Val Glu Lys Ser

595 600 605

Gln Val Gly Phe Ile Asp Tyr Ile Val His Pro Leu Trp Glu Thr Trp

610 615 620

Ala Asp Leu Val His Pro Asp Ala Gln Asp Ile Leu Asp Thr Leu Glu

625 630 635 640

Asp Asn Arg Glu Trp Tyr Gln Ser Thr Ile Pro Gln Ser Pro Ser Pro
645 650 655

Ala Pro Asp Asp Pro Glu Glu Gly Arg Gln Gly Gln Thr Glu Lys Phe
660 665 670

Gln Phe Glu Leu Thr Leu Glu Glu Asp Gly Glu Ser Asp Thr Glu Lys
675 680 685

Asp Ser Gly Ser Gln Val Glu Glu Asp Thr Ser Cys Ser Asp Ser Lys
690 695 700

Thr Leu Cys Thr Gln Asp Ser Glu Ser Thr Glu Ile Pro Leu Asp Glu
705 710 715 720

Gln Val Glu Glu Glu Ala Val Gly Glu Glu Glu Ser Gln Pro Glu
725 730 735

Ala Cys Val Ile Asp Asp Arg Ser Pro Asp Thr
740 745

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Met Ala Phe Val Trp Asp Pro Leu Gly Ala Thr Val Pro Gly Pro Ser

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Thr Arg Ala Lys Ser Arg Leu Arg Phe Ser Lys Ser Tyr Ser Lys Leu
            20                  25                  30
Phe Asp Val Asp Asn Gly Thr Ser Ala Gly Arg Ser Pro Leu Asp Pro
        35                  40                  45
Met Thr Ser Pro Gly Ser Gly Leu Ile Leu Gln Ala Asn Phe Val His
    50                  55                  60
Ser Gln Arg Arg Glu Ser Phe Leu Tyr Arg Ser Asp Ser Asp Tyr Asp
65                  70                  75                  80
Leu Ser Pro Lys Ser Met Ser Arg Asn Ser Ser Ile Ala Ser Asp Ile
                85                  90                  95
His Gly Asp Asp Leu Ile Val Thr Pro Phe Ala Gln Val Leu Ala Ser
            100                 105                 110
Leu Arg Thr Val Arg Asn Asn Phe Ala Ala Leu Thr Asn Leu Gln Asp
        115                 120                 125
Arg Ala Pro Ser Lys Arg Ser Pro Met Cys Asn Gln Pro Ser Ile Asn
    130                 135                 140
Lys Ala Thr Ile Thr Glu Glu Ala Tyr Gln Lys Leu Ala Ser Glu Thr
145                 150                 155                 160
Leu Glu Glu Leu Asp Trp Cys Leu Asp Gln Leu Glu Thr Leu Gln Thr
                165                 170                 175
Arg His Ser Val Ser Glu Met Ala Ser Asn Lys Phe Lys Arg Met Leu
            180                 185                 190
Asn Arg Glu Leu Thr His Leu Ser Glu Met Ser Arg Ser Gly Asn Gln
        195                 200                 205
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Val Ser Glu Phe Ile Ser Asn Thr Phe Leu Asp Lys Gln His Glu Val
210 215 220
Glu Ile Pro Ser Pro Thr Gln Lys Glu Lys Glu Lys Lys Lys Arg Pro
225 230 235 240
Met Ser Gln Ile Ser Gly Val Lys Lys Leu Met His Ser Ser Ser Leu
245 250 255
Thr Asn Ser Ser Ile Pro Arg Phe Gly Val Lys Thr Glu Gln Glu Asp
260 265 270
Val Leu Ala Lys Glu Leu Glu Asp Val Asn Lys Trp Gly Leu His Val
275 280 285
Phe Arg Ile Ala Glu Leu Ser Gly Asn Arg Pro Leu Thr Val Ile Met
290 295 300
His Thr Ile Phe Gln Glu Arg Asp Leu Leu Lys Thr Phe Lys Ile Pro
305 310 315 320
Val Asp Thr Leu Ile Thr Tyr Leu Met Thr Leu Glu Asp His Tyr His
325 330 335
Ala Asp Val Ala Tyr His Asn Asn Ile His Ala Ala Asp Val Val Gln
340 345 350
Ser Thr His Val Leu Leu Ser Thr Pro Ala Leu Glu Ala Val Phe Thr
355 360 365
Asp Leu Glu Ile Leu Ala Ala Ile Phe Ala Ser Ala Ile His Asp Val
370 375 380
Asp His Pro Gly Val Ser Asn Gln Phe Leu Ile Asn Thr Asn Ser Glu
385 390 395 400
Leu Ala Leu Met Tyr Asn Asp Ser Ser Val Leu Glu Asn His His Leu

405 410 415

Ala Val Gly Phe Lys Leu Leu Gln Glu Glu Asn Cys Asp Ile Phe Gln

420 425 430

Asn Leu Thr Lys Lys Gln Arg Gln Ser Leu Arg Lys Met Val Ile Asp

435 440 445

Ile Val Leu Ala Thr Asp Met Ser Lys His Met Asn Leu Leu Ala Asp

450 455 460

Leu Lys Thr Met Val Glu Thr Lys Lys Val Thr Ser Ser Gly Val Leu

465 470 475 480

Leu Leu Asp Asn Tyr Ser Asp Arg Ile Gln Val Leu Gln Asn Met Val

485 490 495

His Cys Ala Asp Leu Ser Asn Pro Thr Lys Pro Leu Gln Leu Tyr Arg

500 505 510

Gln Trp Thr Asp Arg Ile Met Glu Glu Phe Phe Arg Gln Gly Asp Arg

515 520 525

Glu Arg Glu Arg Gly Met Glu Ile Ser Pro Met Cys Asp Lys His Asn

530 535 540

Ala Ser Val Glu Lys Ser Gln Val Gly Phe Ile Asp Tyr Ile Val His

545 550 555 560

Pro Leu Trp Glu Thr Trp Ala Asp Leu Val His Pro Asp Ala Gln Asp

565 570 575

Ile Leu Asp Thr Leu Glu Asp Asn Arg Glu Trp Tyr Gln Ser Thr Ile

580 585 590

Pro Gln Ser Pro Ser Pro Ala Pro Asp Asp Pro Glu Glu Gly Arg Gln

595 600 605

Gly Gln Thr Glu Lys Phe Gln Phe Glu Leu Thr Leu Glu Glu Asp Gly
610 615 620
Glu Ser Asp Thr Glu Lys Asp Ser Gly Ser Gln Val Glu Glu Asp Thr
625 630 635 640
Ser Cys Ser Asp Ser Lys Thr Leu Cys Thr Gln Asp Ser Glu Ser Thr
645 650 655
Glu Ile Pro Leu Asp Glu Gln Val Glu Glu Glu Ala Val Gly Glu Glu
660 665 670
Glu Glu Ser Gln Pro Glu Ala Cys Val Ile Asp Asp Arg Ser Pro Asp
675 680 685
Thr

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Glu Glu Thr Leu His Ser Ser Asn Glu Glu Glu Asp Pro Phe Arg Gly

-20 25 30

Met Glu Pro Tyr Leu Val Arg Arg Leu Ser Cys Arg Asn Ile Gln Leu

35 40 45

Pro Pro Leu Ala Phe Arg Gln Leu Glu Gln Ala Asp Leu Lys Ser Glu

50 55 60

Ser Glu Asn Ile Gln Arg Pro Thr Ser Leu Pro Leu Lys Ile Leu Pro

65 70 75 80

Leu Ile Ala Ile Thr Ser Ala Glu Ser Ser Gly Lys Leu Phe Asp Val

85 90 95

Asp Asn Gly Thr Ser Ala Gly Arg Ser Pro Leu Asp Pro Met Thr Ser

100 105 110

Pro Gly Ser Gly Leu Ile Leu Gln Ala Asn Phe Val His Ser Gln Arg

115 120 125

Arg Glu Ser Phe Leu Tyr Arg Ser Asp Ser Asp Tyr Asp Leu Ser Pro

130 135 140

Lys Ser Met Ser Arg Asn Ser Ser Ile Ala Ser Asp Ile His Gly Asp

145 150 155 160

Asp Leu Ile Val Thr Pro Phe Ala Gln Val Leu Ala Ser Leu Arg Thr

165 170 175

Val Arg Asn Asn Phe Ala Ala Leu Thr Asn Leu Gln Asp Arg Ala Pro

180 185 190

Ser Lys Arg Ser Pro Met Cys Asn Gln Pro Ser Ile Asn Lys Ala Thr

195 200 205

Ile Thr Glu Glu Ala Tyr Gln Lys Leu Ala Ser Glu Thr Leu Glu Glu

210 215 220

Leu Asp Trp Cys Leu Asp Gln Leu Glu Thr Leu Gln Thr Arg His Ser
225 230 235 240
Val Ser Glu Met Ala Ser Asn Lys Phe Lys Arg Met Leu Asn Arg Glu
245 250 255
Leu Thr His Leu Ser Glu Met Ser Arg Ser Gly Asn Gln Val Ser Glu
260 265 270
Phe Ile Ser Asn Thr Phe Leu Asp Lys Gln His Glu Val Glu Ile Pro
275 280 285
Ser Pro Thr Gln Lys Glu Lys Glu Lys Lys Lys Arg Pro Met Ser Gln
290 295 300
Ile Ser Gly Val Lys Lys Leu Met His Ser Ser Ser Leu Thr Asn Ser
305 310 315 320
Ser Ile Pro Arg Phe Gly Val Lys Thr Glu Gln Glu Asp Val Leu Ala
325 330 335
Lys Glu Leu Glu Asp Val Asn Lys Trp Gly Leu His Val Phe Arg Ile
340 345 350
Ala Glu Leu Ser Gly Asn Arg Pro Leu Thr Val Ile Met His Thr Ile
355 360 365
Phe Gln Glu Arg Asp Leu Leu Lys Thr Phe Lys Ile Pro Val Asp Thr
370 375 380
Leu Ile Thr Tyr Leu Met Thr Leu Glu Asp His Tyr His Ala Asp Val
385 390 395 400
Ala Tyr His Asn Asn Ile His Ala Ala Asp Val Val Gln Ser Thr His
405 410 415
Val Leu Leu Ser Thr Pro Ala Leu Glu Ala Val Phe Thr Asp Leu Glu

420 425 430

Ile Leu Ala Ala Ile Phe Ala Ser Ala Ile His Asp Val Asp His Pro

435 440 445

Gly Val Ser Asn Gln Phe Leu Ile Asn Thr Asn Ser Glu Leu Ala Leu

450 455 460

Met Tyr Asn Asp Ser Ser Val Leu Glu Asn His His Leu Ala Val Gly

465 470 475 480

Phe Lys Leu Leu Gln Glu Glu Asn Cys Asp Ile Phe Gln Asn Leu Thr

485 490 495

Lys Lys Gln Arg Gln Ser Leu Arg Lys Met Val Ile Asp Ile Val Leu

500 505 510

Ala Thr Asp Met Ser Lys His Met Asn Leu Leu Ala Asp Leu Lys Thr

515 520 525

Met Val Glu Thr Lys Lys Val Thr Ser Ser Gly Val Leu Leu Leu Asp

530 535 540

Asn Tyr Ser Asp Arg Ile Gln Val Leu Gln Asn Met Val His Cys Ala

545 550 555 560

Asp Leu Ser Asn Pro Thr Lys Pro Leu Gln Leu Tyr Arg Gln Trp Thr

565 570 575

Asp Arg Ile Met Glu Glu Phe Phe Arg Gln Gly Asp Arg Glu Arg Glu

580 585 590

Arg Gly Met Glu Ile Ser Pro Met Cys Asp Lys His Asn Ala Ser Val

595 600 605

Glu Lys Ser Gln Val Gly Phe Ile Asp Tyr Ile Val His Pro Leu Trp

610 615 620

Glu Thr Trp Ala Asp Leu Val His Pro Asp Ala Gln Asp Ile Leu Asp
625 630 635 640
Thr Leu Glu Asp Asn Arg Glu Trp Tyr Gln Ser Thr Ile Pro Gln Ser
645 650 655
Pro Ser Pro Ala Pro Asp Asp Pro Glu Glu Gly Arg Gln Gly Gln Thr
660 665 670
Glu Lys Phe Gln Phe Glu Leu Thr Leu Glu Glu Asp Gly Glu Ser Asp
675 680 685
Thr Glu Lys Asp Ser Gly Ser Gln Val Glu Glu Asp Thr Ser Cys Ser
690 695 700
Asp Ser Lys Thr Leu Cys Thr Gln Asp Ser Glu Ser Thr Glu Ile Pro
705 710 715 720
Leu Asp Glu Gln Val Glu Glu Glu Ala Val Gly Glu Glu Glu Glu Ser
725 730 735
Gln Pro Glu Ala Cys Val Ile Asp Asp Arg Ser Pro Asp Thr
740 745 750

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Met Ser Ile Ile Met Lys Pro Arg Ser Arg Ser Thr Ser Ser Leu Arg
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Thr Ala Glu Ala Lys Leu Phe Asp Val Asp Asn Gly Thr Ser Ala Gly
20 25 30
Arg Ser Pro Leu Asp Pro Met Thr Ser Pro Gly Ser Gly Leu Ile Leu
35 40 45
Gln Ala Asn Phe Val His Ser Gln Arg Arg Glu Ser Phe Leu Tyr Arg
50 55 60
Ser Asp Ser Asp Tyr Asp Leu Ser Pro Lys Ser Met Ser Arg Asn Ser
65 70 75 80
Ser Ile Ala Ser Asp Ile His Gly Asp Asp Leu Ile Val Thr Pro Phe
85 90 95
Ala Gln Val Leu Ala Ser Leu Arg Thr Val Arg Asn Asn Phe Ala Ala
100 105 110
Leu Thr Asn Leu Gln Asp Arg Ala Pro Ser Lys Arg Ser Pro Met Cys
115 120 125
Asn Gln Pro Ser Ile Asn Lys Ala Thr Ile Thr Glu Glu Ala Tyr Gln
130 135 140
Lys Leu Ala Ser Glu Thr Leu Glu Glu Leu Asp Trp Cys Leu Asp Gln
145 150 155 160
Leu Glu Thr Leu Gln Thr Arg His Ser Val Ser Glu Met Ala Ser Asn
165 170 175
Lys Phe Lys Arg Met Leu Asn Arg Glu Leu Thr His Leu Ser Glu Met
180 185 190
Ser Arg Ser Gly Asn Gln Val Ser Glu Phe Ile Ser Asn Thr Phe Leu

195 200 205

Asp Lys Gln His Glu Val Glu Ile Pro Ser Pro Thr Gln Lys Glu Lys

210 215 220

Glu Lys Lys Lys Arg Pro Met Ser Gln Ile Ser Gly Val Lys Lys Leu

225 230 235 240

Met His Ser Ser Ser Leu Thr Asn Ser Ser Ile Pro Arg Phe Gly Val

245 250 255

Lys Thr Glu Gln Glu Asp Val Leu Ala Lys Glu Leu Glu Asp Val Asn

260 265 270

Lys Trp Gly Leu His Val Phe Arg Ile Ala Glu Leu Ser Gly Asn Arg

275 280 285

Pro Leu Thr Val Ile Met His Thr Ile Phe Gln Glu Arg Asp Leu Leu

290 295 300

Lys Thr Phe Lys Ile Pro Val Asp Thr Leu Ile Thr Tyr Leu Met Thr

305 310 315 320

Leu Glu Asp His Tyr His Ala Asp Val Ala Tyr His Asn Asn Ile His

325 330 335

Ala Ala Asp Val Val Gln Ser Thr His Val Leu Leu Ser Thr Pro Ala

340 345 350

Leu Glu Ala Val Phe Thr Asp Leu Glu Ile Leu Ala Ala Ile Phe Ala

355 360 365

Ser Ala Ile His Asp Val Asp His Pro Gly Val Ser Asn Gln Phe Leu

370 375 380

Ile Asn Thr Asn Ser Glu Leu Ala Leu Met Tyr Asn Asp Ser Ser Val

385 390 395 400

Leu Glu Asn His His Leu Ala Val Gly Phe Lys Leu Leu Gln Glu Glu
405 410 415
Asn Cys Asp Ile Phe Gln Asn Leu Thr Lys Lys Gln Arg Gln Ser Leu
420 425 430
Arg Lys Met Val Ile Asp Ile Val Leu Ala Thr Asp Met Ser Lys His
435 440 445
Met Asn Leu Leu Ala Asp Leu Lys Thr Met Val Glu Thr Lys Lys Val
450 455 460
Thr Ser Ser Gly Val Leu Leu Leu Asp Asn Tyr Ser Asp Arg Ile Gln
465 470 475 480
Val Leu Gln Asn Met Val His Cys Ala Asp Leu Ser Asn Pro Thr Lys
485 490 495
Pro Leu Gln Leu Tyr Arg Gln Trp Thr Asp Arg Ile Met Glu Glu Phe
500 505 510
Phe Arg Gln Gly Asp Arg Glu Arg Glu Arg Gly Met Glu Ile Ser Pro
515 520 525
Met Cys Asp Lys His Asn Ala Ser Val Glu Lys Ser Gln Val Gly Phe
530 535 540
Ile Asp Tyr Ile Val His Pro Leu Trp Glu Thr Trp Ala Asp Leu Val
545 550 555 560
His Pro Asp Ala Gln Asp Ile Leu Asp Thr Leu Glu Asp Asn Arg Glu
565 570 575
Trp Tyr Gln Ser Thr Ile Pro Gln Ser Pro Ser Pro Ala Pro Asp Asp
580 585 590
Pro Glu Glu Gly Arg Gln Gly Gln Thr Glu Lys Phe Gln Phe Glu Leu

595 600 605

Thr Leu Glu Glu Asp Gly Glu Ser Asp Thr Glu Lys Asp Ser Gly Ser

610 615 620

Gln Val Glu Glu Asp Thr Ser Cys Ser Asp Ser Lys Thr Leu Cys Thr

625 630 635 640

Gln Asp Ser Glu Ser Thr Glu Ile Pro Leu Asp Glu Gln Val Glu Glu

645 650 655

Glu Ala Val Gly Glu Glu Glu Glu Ser Gln Pro Glu Ala Cys Val Ile

660 665 670

Asp Asp Arg Ser Pro Asp Thr

675

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Met Glu Ala Glu Gly Ser Ser Ala Pro Ala Arg Ala Gly Ser Gly Glu
1 5 10 15

Gly Ser Asp Ser Ala Gly Gly Ala Thr Leu Lys Ala Pro Lys His Leu
20 25 30

Trp Arg His Glu Gln His His Gln Tyr Pro Leu Arg Gln Pro Gln Phe
35 40 45

Arg Leu Leu His Pro His His His Leu Pro Pro Pro Pro Pro Pro Ser
50 55 60

Pro Gln Pro Gln Pro Gln Cys Pro Leu Gln Pro Pro Pro Pro Pro Pro

65 70 75 80

Leu Pro Pro Pro Pro Pro Pro Pro Gly Ala Ala Arg Gly Arg Tyr Ala

85 90 95

Ser Ser Gly Ala Thr Gly Arg Val Arg His Arg Gly Tyr Ser Asp Thr

100 105 110

Glu Arg Tyr Leu Tyr Cys Arg Ala Met Asp Arg Thr Ser Tyr Ala Val

115 120 125

Glu Thr Gly His Arg Pro Gly Leu Lys Lys Ser Arg Met Ser Trp Pro

130 135 140

Ser Ser Phe Gln Gly Leu Arg Arg Phe Asp Val Asp Asn Gly Thr Ser

145 150 155 160

Ala Gly Arg Ser Pro Leu Asp Pro Met Thr Ser Pro Gly Ser Gly Leu

165 170 175

Ile Leu Gln Ala Asn Phe Val His Ser Gln Arg Arg Glu Ser Phe Leu

180 185 190

Tyr Arg Ser Asp Ser Asp Tyr Asp Leu Ser Pro Lys Ser Met Ser Arg

195 200 205

Asn Ser Ser Ile Ala Ser Asp Ile His Gly Asp Asp Leu Ile Val Thr

210 215 220

Pro Phe Ala Gln Val Leu Ala Ser Leu Arg Thr Val Arg Asn Asn Phe

225 230 235 240

Ala Ala Leu Thr Asn Leu Gln Asp Arg Ala Pro Ser Lys Arg Ser Pro

245 250 255

Met Cys Asn Gln Pro Ser Ile Asn Lys Ala Thr Ile Thr Glu Glu Ala

260 265 270

Tyr Gln Lys Leu Ala Ser Glu Thr Leu Glu Glu Leu Asp Trp Cys Leu
275 280 285
Asp Gln Leu Glu Thr Leu Gln Thr Arg His Ser Val Ser Glu Met Ala
290 295 300
Ser Asn Lys Phe Lys Arg Met Leu Asn Arg Glu Leu Thr His Leu Ser
305 310 315 320
Glu Met Ser Arg Ser Gly Asn Gln Val Ser Glu Phe Ile Ser Asn Thr
325 330 335
Phe Leu Asp Lys Gln His Glu Val Glu Ile Pro Ser Pro Thr Gln Lys
340 345 350
Glu Lys Glu Lys Lys Lys Arg Pro Met Ser Gln Ile Ser Gly Val Lys
355 360 365
Lys Leu Met His Ser Ser Ser Leu Thr Asn Ser Ser Ile Pro Arg Phe
370 375 380
Gly Val Lys Thr Glu Gln Glu Asp Val Leu Ala Lys Glu Leu Glu Asp
385 390 395 400
Val Asn Lys Trp Gly Leu His Val Phe Arg Ile Ala Glu Leu Ser Gly
405 410 415
Asn Arg Pro Leu Thr Val Ile Met His Thr Ile Phe Gln Glu Arg Asp
420 425 430
Leu Leu Lys Thr Phe Lys Ile Pro Val Asp Thr Leu Ile Thr Tyr Leu
435 440 445
Met Thr Leu Glu Asp His Tyr His Ala Asp Val Ala Tyr His Asn Asn
450 455 460
Ile His Ala Ala Asp Val Val Gln Ser Thr His Val Leu Leu Ser Thr

465 470 475 480

Pro Ala Leu Glu Ala Val Phe Thr Asp Leu Glu Ile Leu Ala Ala Ile

485 490 495

Phe Ala Ser Ala Ile His Asp Val Asp His Pro Gly Val Ser Asn Gln

500 505 510

Phe Leu Ile Asn Thr Asn Ser Glu Leu Ala Leu Met Tyr Asn Asp Ser

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Ser Val Leu Glu Asn His His Leu Ala Val Gly Phe Lys Leu Leu Gln

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Glu Glu Asn Cys Asp Ile Phe Gln Asn Leu Thr Lys Lys Gln Arg Gln

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Ser Leu Arg Lys Met Val Ile Asp Ile Val Leu Ala Thr Asp Met Ser

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Lys His Met Asn Leu Leu Ala Asp Leu Lys Thr Met Val Glu Thr Lys

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Lys Val Thr Ser Ser Gly Val Leu Leu Leu Asp Asn Tyr Ser Asp Arg

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Ile Gln Val Leu Gln Asn Met Val His Cys Ala Asp Leu Ser Asn Pro

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Glu Phe Phe Arg Gln Gly Asp Arg Glu Arg Glu Arg Gly Met Glu Ile

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Ser Pro Met Cys Asp Lys His Asn Ala Ser Val Glu Lys Ser Gln Val

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